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(54) Tocopherol cyclase.

(57) The invention concerns a novel enzyme, namely a cyclase, and more specifically, a tocopherol cyclase in homogeneous, pure form which acts on phytyl benzoquinol derivatives to produce R',R',R'-tocopherols enantioselectively.

EP 0 531 639 A2

The invention concerns a novel enzyme, namely a cyclase, and more specifically, a tocopherol cyclase in homogeneous, pure form which acts on phytyl benzoquinol derivatives to produce R',R',R'-tocopherols enantioselectively.

The enzyme can be characterized by the following properties:

molecular weight: 48 kD - 50 kD

and containing at least one of the following amino acid sequences:

H_2N-(Pro)-Leu-Tyr-Ile-Pro-X-Val-(Glu)-(Pro)-(Met)-(Tyr)-(Asp)-(Asp)-(Ala)-(Ala)-(Phe)-(Thr);

X-Leu-Ala-Pro-Val-Gln-(Ser)-Pro;

(Gly)-Leu-Asp-Leu-Ala-Pro;

X-Val-Gln-Leu-(Asp)-(Ser)-Asp-Gly-Glu-(Thr)-Val;

X-Leu-(Pro)-Val-;

X being an amino acid residue not determinable and

( ) indicating uncertainty.

It is preferably of algal or plant origin, more precisely, it occurs in particular in green algae, for example in Dunaliella salina or, preferably, Chlorella protothecoides, or in wheat leaves.

A further embodiment of the present invention is the production of the novel enzyme.

Such production comprises the following isolation and purification steps:

a) centrifugation
b) gel filtration
c) hydrophobic interaction chromatography
d) ion exchange chromatography
e) reverse phase chromatography.

The identification encompasses:

f) SDS-PAGE electrophoresis
g) electroblotting and
h) amino acid sequencing.

The purified enzyme can thus be characterized by its above amino acid sequence.

Growth of cells, cell breakage

The photosynthetic organisms can be grown in the absence of light (heterotrophic growth) or in the presence of light (autotrophic growth). In the case of heterotrophic growth, an organic carbon source is required.

The cells may be cultured in an aqueous medium supplemented with appropriate nutrients under aerobic conditions. The cultivation may be conducted at pH of about 4.0 to about 8.0, preferably from about 4.5 to about 6.5. The cultivation period varies depending upon the microorganisms and nutrient medium to be used, preferably about 10 to about 100 hours. A preferred temperature range for carrying out for the cultivation is from about 10 °C to about 40 °C, preferably from about 25 °C to about 35 °C.

It is usually required that the culture medium contains nutrients such as: assimilable carbon sources (heterotrophic only) such as D-glucose, sodium acetate, etc.; digestible nitrogen sources such as organic substances, for example, peptone, yeast extract, etc.; and inorganic substances, for example, ammonium sulfate, ammonium chloride and potassium nitrite; vitamins and trace elements.

The enzyme activity is absent in logarithmically growing cells and starts to appear only at the beginning of stationary phase where it rapidy rises to a maximum value, then levels off (Fig. 3). Cells can thus be harvested at maximal cell density. The cell yield is typically around 20 g/l (wet weight) under heterotrophic conditions. Markedly higher levels of enzyme activity are observed when the cells are grown autotrophically. However much lower cell densities ensue when cells are grown autotrophically.

During fermentation, tocopherol cyclase activity is measured by harvesting a fixed number of cells, e.g. about $1x10^9$, and lysing them mechanically; the supernatant obtained by centrifuging the lysate at 100000xg for 30 min is mixed with radiolabelled 2,3-dimethyl-6-phytylhydroquinone and the mixture incubated for 12-

15 hr at 30 °C under reducing conditions. Radiolabelled hydroquinone and gamma-tocopherol are extracted into hexene, separated by HPLC and quantitated by radiodetection.

A convenient scheme for the isolation and purification of the enzyme after the cultivation of cells is as follows:

1) cells are harvested from the fermentation broth by centrifugation;

2) the cells are suspended in the buffer solution and disrupted mechanically, i.e. lysed by means of a homogenizer, to give a disrupted suspension. A convenient buffer is of the phosphate type;

3) the soluble enzyme is isolated and purified from the soluble extract of the disrupted cells following removal of cell membranes by centrifugation, as described below.

Centrifugation

Cells containing tocopherol cyclase activity are conveniently harvested, e.g. by continous centrifugation and stored at -20 °C until required. Enzyme activity remains stable over several months. Cells to be used are thawed into an excess, e.g. an 2.5 fold excess, of potassium phosphate buffer, pH 7. The suspension is lysed mechanically, e.g. by rotation with glass beads and the resulting lysate centrifuged to remove cell debris. The supernatant obtained by centrifugation is made ca. 60% saturated with ammonium sulphate in order to precipitate the proteins. The resulting precipitate is removed by centrifugation and resuspended in a small volume of the potassium phosphate buffer.

Gel filtration (molecular sizing chromatography)

With this technique proteins are separated according to molecular weight. The preferred matrix is one which allows separation of proteins in the molecular weight range 5000-250000 D, e.g. Sephacryl S-200HR. In this instance the gel filtration step serves to separate the tocopherol cyclase (activity) from the bulk of colored components in the cell extract. In the present sequence of steps, it serves thus as a prelimary purification step.

The material to be chromatographed is loaded onto the column in aqueous buffer at pH 6.5-7. A convenient buffer is of the phosphate type. After chromatography, those fractions containing tocopherol cyclase activity are selected for further purification.

Since the activity is contained in a large volume, concentration of the material is necessary before the next purification step. The active fractions can be conveniently concentrated on a (small) bed of a strong anion exchanger, such as Q-Sepharose. The material is loaded in dilute salt solution whereupon it binds tightly to the anion exchanger; it can then be eluted with a small volume of concentrated salt solution. Ca. 0.4-0.8 M alkali halide is appropriate.

Hydrophobic interaction chromatography

As is known, this technique is based on the interaction betwen the aliphatic chains on the adsorbent and the corresponding hydrophobic regions on the surface of the proteins, causing proteins to bind. The adsorbents preferably used are the commercially available C_4 linear aliphatic chain materials, e.g. Butyl-Sepharose, since the cyclase to be purified is considered to be medium to weakly hydrophobic.

The material to be separated is adsorbed onto the column in high salt solution and is eluted with a reverse and linear salt gradient.

The preferred salt is ammonium sulfate. The initial salt concentration is ca. 600-700 mM and the final salt concentration is ca. 0-20 mM.

After this purification step, the product is conveniently dialyzed in order to remove the ammonium sulphate.

Ion-exchange chromatography

In the present case anion-exchange is the method of choice: thus the negatively charged regions of proteins are exploited for adsorption to a positively charged matrix.

A strong anion exchanger containing quarternary amines as charged groups is the preferred exchanger (e.g. Mono Q). Proteins are adsorbed in a buffer containing little salt, at pH 7, and eluted with a gradient of buffer containing high salt concentrations, e.g. ca. 1 M of an alkali halide, e.g. 1M KCl.

The initial salt concentration is ca. 0-20 mm. The final salt concentration is ca. 300-500 mM.

The gradient is preferably linear.

Reverse phase chromatography

This term is used in the present context to refer to the method c), with the difference that organic solvents and a somewhat more hydrophobic adsorbent are used. The proteins are absorbed onto a column in a buffer solution and eluted with a increasing gradient of the organic solvent. A convenient buffer is of the phosphate type. A convenient column material is Pro RPC, a macro-porous microparticulate silica with bonded C_1/C_8 groups.

Convenient parameters are:

initial solvent: ca. 0-10% isopropanol

final solvent: ca. 30-50% isopropanol

This purification steps allows the separation of two cyclases of molecular weights of 48 and 50 kD from remaining impurities.

SDS-PAGE and Electroblotting

As is known, the mobility of proteins in polyacrylamide gels in the presence of SDS is a linear function of the logarithm of their molecular weight.

The method thus allows the purity of a protein to be analysed or it can also be used as a purification method per se.

In the present case SDS-PAGE is used essentially as an analytical step but, followed by electroblotting, it is also a convenient method to prepare the cyclase in a suitable form for N-terminal sequence analysis.

After electrophoresis, the gel is conveniently electroblotted to a PVDF (polyvinylidene fluoride) membrane and the transferred proteins further characterised.

The enzymes thus purified can now be characterized by state-of-the-art peptide chemistry methods, such as N-terminal amino acid sequencing with enzymatic or chemical peptide cleavage. Fragments obtained by enzymatic or chemical cleavage can be separated according to usual methods, such as HPLC, and can themselves be subjected to further N-terminal sequencing. The experimental details are outlined in the Examples.

Assay for the novel cyclase

The assay for the novel cyclase described for fermentation analysis above in Examples 1 and 2 below was also used to monitor the cyclase activity following:

gel filtration

hydrophobic interaction chromatography

ion exchange chromatography

reverse phase chromatography.

It makes use of the ability of the novel cyclase to catalyze the reaction of 2,3-dimethyl-6-phytyl-hydroquinone to R',R',R',γ-tocopherol.

Example 1

Identification of tocopherol cyclase activity in wheat leaves.

40 g of wheat grains (variety Fidel) are washed in 70% ethanol for 5 minutes, then rinsed in H_2O; the grains are then aereated for 2-4 hrs in H_2O. They are subsequently distributed evenly on wet filter paper in a 30x50 cm seed tray and incubated at 30°C in the dark for 3 days. The germinated grains are then brought into the light and allowed to grow for ca. 2 weeks at ambient light and temperature.

The leaves from one tray of plants are rapidly frozen by immersion in liquid N_2 and immediately ground into a fine powder with pestle and mortar. The powder is then mixed with 250 ml of a buffer comprising 30mM potassium phosphate pH 7, 1mM dithiothreitol, 10% sucrose, 5mM sodium ascorbate and stirred at 4°C for 1 hr.

The suspension is centrifuged at 2000xg for 10 minutes, then at 40000xg for 1 hr. This high speed supernatant is then assayed for tocopherol cyclase activity by adding the substrate (3H,^{14}C-)2,3-dimethyl-6-phytyl-hydroquinone and incubating at 30°C for 15 hr. The incubation mixture is extracted with hexane:methanol (1:4) and the hexane phase injected onto a 220x4.6mm Spheri-5 silica HPLC column (Kontron Analytics); the column is eluted isocratically with hexane:isopropanol (99:1). Under these conditons the dimethylphytylbenzoquinone is not retained by the column and elutes at ca. 3 min; the hydroquinone form

of the substrate elutes at ca. 6 min and gamma-tocopherol at ca. 5 min. The chromatogram shown in Figure 1 contains a peak corresponding to gamma-tocopherol, indicating the presence of an enzyme with tocopherol cyclase activity in this wheat leaf extract.

Example 2

Identification of tocopherol cyclase activity in Dunaliella salina.

A Dunaliella salina inoculum was obtained from Western Biotechnology Ltd, Austrialia. Cells are cultured in 2 l Erlenmeyer flasks containing 1 l of medium at ambient temperature and light. The culture medium is modified Johnson's medium (Johnson et al., J. Bacteriology 95 (1968) pp 1461-1468) containing 4.3M NaCl. Cultures are assessed to be in stationary phase when they become visibly orange (senescent cultures produce carotenoids in the presence of high salt concentrations), which corresponds to a cell count of ca. $5x10^5$ cells/ml.

Cells are pelleted by centrifugation and resuspended at a concentration of ca. $2x10^8$/ml in 30mM potassium phosphate buffer pH 7, containing 10% sucrose, 4mM $MgSO_4$, 0.2mM EDTA. The suspension is then sonicated briefly, centrifuged at 100000xg for 1 hr and the resulting supernatant diluted 10-fold in 100mM potassium phosphate pH 7 for assay. The assay is performed as in Example 1. The chromatogram of Figure 2 shows the presence of a peak at ca. 5 min which corresponds to gamma-tocopherol. Dunaliella salina would therefore appear to contain a tocopherol cyclase.

Example 3

Growth of Chlorella protothecoides and identification of tocopherol cyclase activity.

Chlorella protothecoides Krüger 1894 (CCAP 211/8D) was obtained from the Culture Collection of Algae and Protozoa in Cumbria, England. Cells are grown in 10 l blade-stirred glass fermentors under essentially heterotrophic growth conditions (fermentors are exposed to dim laboratory light during the day). The fermentation medium is as follows:

yeast extract (BBL)	100 g
peptone	100 g
glucose	200 g

made up to 10 l with distilled H_2O.

Fermentation is carried out at 30 °C and at a stir rate of 160 rpm. A sample of cells is withdrawn daily for tocopherol cyclase activity assay. After completion of fermentation the cells are centrifuged and stored as pellets at -20 °C until required for purification.

The assay is prepared by centrifuging down and resuspending $1x10^9$ cells in 5 ml of 30mM potassium phosphate pH 7, 10% (wt/vol) glycerol, 0.01% (wt/vol) lauryl maltoside. The suspension is mixed with 7 g of 0.5 mm glass beads, frozen, then shaken for 3 min in a Braun Homogeniser. The lysate is subsequently centrifuged at 100000xg for 40 min and 1 ml of the resulting supernatant taken for assay. The assay is performed as in Example 1. Relative activity of the enzyme is expressed as the percentage of total radioactivity incorporated into gamma-tocopherol by $2x10^8$ cells. Figure 3 shows growth versus activity for C. protothecoides. Tocopherol cyclase activity is only measurable once the cells start to enter stationary phase and cells are thus harvested after 5 days in stationary phase. The cell yield is ca. 20 g/l (wet weight).

Example 4

Purification of Tocopherol cyclase from C. protothecoides.

a. Cell lysis and preparation of a crude extract.

300 g of cells (see Example 3) are thawed and suspended in 750 ml of 30mM potassium phosphate pH 7, 10% (wt/vol) glycerol, 1mM phenylmethylsulphonyl fluoride (PMSF; a protease inhibitor), 5mM sodium ascorbate. The cells are then broken mechanically by rotation with glass beads, for example with a Dyno-Mill cell disintegrator (W. Bachofen Maschinenfabrik AG, Basel). The suspension is pumped continuously at

a rate of ca. 30 ml/min through the Dyno-Mill operating at 2000 rpm. The cell suspension and the resulting lysate are kept on ice. The lysate is subsequently centrifuged at 10000xg for 15 min to remove unbroken cells and cell debris. The supernatant from this centrifugation step is then centrifuged at 100000xg for 1 hr. The second supernatant is then made 60% saturated with ammonium sulphate and stirred for 30 min at ambient temperature. The precipitate is removed by centrifugation and resuspended in 250 ml of 20mM potassium phosphate pH 7, 10% glycerol. This material is then divided into 50 ml portions and stored at -20 °C until required.

b. Gel filtration on Sephacryl S-200HR.

A 50 ml portion of crude extract is thawed and loaded onto a molecular sizing column, for example a 5x70 cm Sephacryl S-200HR column (Pharmacia LKB), equilibrated in 20mM potassium phosphate pH 7, 10% glycerol and pumped at a rate of 200 ml/hr. Fractions of 10 ml are collected and assayed for tocopherol cyclase activity. Active fractions are pooled and stored at -20 °C until required.

c. Concentration on Q-Sepharose.

The active pool from above is thawed and diluted 1:1 with 10% glycerol. This protein solution can then be concentrated on a small bed of the strong anion exchange resin, Q-Sepharose (Pharmacia LKB). Thus the material is passed over a 2.5x1 cm bed of Q-Sepharose equilibrated in 10mM potassium phosphate pH 7, 10% glycerol. The column is then washed with the same buffer and eluted with 15 ml of 15 mM potassium phosphate pH 7, 10% glycerol, 400mM NaCl.

d. Chromatography on Butyl-Sepharose.

The eluate from above is made 15% saturated with ammonium sulphate. This material is then further purified by hydrophobic interaction chromatography on a C_4 matrix, i.e. Butyl-Sepharose (Pharmacia LKB). It is loaded onto a 1.6x12.5 cm Butyl-Sepharose column equilibrated in 15mM potassium phosphate pH 7, 15% (saturation) ammonium sulphate, 10% glycerol. The column is washed with the same buffer and eluted with a 0-100% gradient of 10% glycerol, 30% ethylene glycol (total gradient volume = 100 ml). Fractions of 5 ml are collected and assayed for tocopherol cyclase activity. Active fractions are pooled.

e. Chromatography on Mono Q.

The active pool from above is made 0.025% (wt/vol) in lauryl maltoside and dialysed for 15 hr at 4 °C against 15mM potassium phosphate pH 7, 10% glycerol in order to remove ammonium sulphate. This material is then loaded on a column of the compact anion exchange resin Mono Q in a HR5/5 column (Pharmacia LKB) which has been equilibrated in 15mM potassium phosphate pH 7, 10% glycerol, 0.025% lauryl maltoside. The column is washed with this buffer and eluted at a flow rate of 1 ml/min with a gradient of 15mM potassium phosphate pH 7, 10% glycerol, 0.025% lauryl maltoside, 1M KCl (Buffer B); the (linear) gradient has the following profile:

0-30% Buffer B	15 ml
30% Buffer B	2 ml
30-50% Buffer B	3 ml
50% Buffer B	2 ml

1 ml fractions are collected and assayed for tocopherol cyclase activity. The active fractions are pooled.

f. Chromatography on ProRPC.

A C1/C8 reverse phase column, i.e. a ProRPC HR5/10 column from Pharmacia LKB is used for further purification. The ProRPC column is equilibrated in 5mM potassium phosphate buffer pH 7. 1 ml of the active pool from above is loaded onto the column. The column is then washed with 10% isopropanol and eluted at 0.5 ml/min with a linear gradient to 50% isopropanol (total gradient volume = 25 ml). Fractions of 0.5 ml are collected and every second assayed for tocopherol cyclase activity. Samples are also withdrawn from every fraction for SDS-PAGE analysis. Figure 4 shows the chromatogram and the results of the activity

measurements; this figure also shows the corresponding SDS-PAGE gel. The cyclase activity is associated with 2 proteins, one of molecular weight ca. 50 kD and the other with a molecular weight of 48 kD.

Example 5

Separation of 48 kD and 50 kD Tocopherol Cyclases for Amino Acid Analysis and Protein Sequence Analysis.

Following chromatography on ProRPC, fractions containing tocopherol cyclase activity are pooled (total volume = 6.5 ml) and concentrated to ca. 350 µl. Of this, 30 µl are removed and the quality and quantity assessed by SDS-PAGE with Coomassie Blue staining. The remaining 320 µl are mixed with 35 µl denaturing buffer containing the reducing agent mercaptoethanol (U.K. Laemmli [1970] Nature 227, 680-685) and heated at 95 °C until the volume reduces to 140 µl. The protein is then electrophoresed on an 8% SDS-PAGE gel. After electrophoresis the gel is used for electroblotting to a polyvinylidene fluoride (PVDF) membrane (Immobilon P, Millipore); blotting is carried out in 10mM 3-(cyclohexylamino)-1-propane sulphonic acid (CAPS) buffer pH 11, 10% (vol/vol) methanol (Matsudaira, P. [1987] J. Biol. Chem. 261, 10035-10038) for 1 hr at 90V and at 4 °C. The PVDF membrane is subsequently stained with 0.5% Ponceau S in 1% acetic acid to visualise the transferred protein. A strip of membrane containing the tocopherol cyclase band is then excised and stored at -20 °C until required.

ProRPC chromatography does not result in the resolution of 48 and 50 kD tocopherol cyclases. Consequently pooled active material contains both forms. However if the protein is subsequently electrophoresed on an 8% SDS-PAGE gel in the absence of mercaptoethanol the 48 and 50 kD proteins resolve from each other (in the presence of mercaptoethanol the two proteins do not resolve). This observation can be utilised to purify the 48 kD tocopherol cyclase for amino acid and protein sequence analysis by the method described above. Thus mercaptoethanol was excluded from the dissociation buffer and, following electroblotting and Ponceau staining of the PVDF membrane, the two proteins were individually excised.

Example 6

Amino Acid Analysis and Amino Acid Sequence Analysis of the Two Tocopherol Cyclases

A. Amino Acid Analysis

The membrane fragments prepared in Example 5 are incubated in the presence of 6N HCl for 24 hr at 110 °C. The hydrolysed amino acids are extracted from the membranes with 2x100 µl formic acid (1 hr incubation at 37 °C) and analysed according to standard procedures on an AminoQuant amino acid analyser (Hewlett Packard). Amino acid analyses (Figure 5) for the 48 kD cyclase and a mixture of the 48 kD and 50 kD proteins show that the two cyclases are closely related.

B. Amino Acid Sequence Analysis

The membrane fragments prepared as in Example 5 are rinsed with water and air-dried. They are then treated with either trypsin or Proteinase K *in situ* and the peptides obtained separated by reverse phase HPLC.

Trypsin cleavage. The membrane piece containing the protein band is placed in an Eppendorf tube containing 1 ml of PVP-30 solution (polyvinylpyrrolidone in 100mM acetic acid) and incubated at 37 °C for 30 min. The PVP-30 solution is then removed and the membrane thoroughly washed with 10 ml of water. It is then cut into small pieces and transferred to a clean Eppendorf tube containing 200 µl of 100mM ammonium bicarbonate pH 8.0. A solution of 10 ng of trypsin in 0.1% trifluoroacetic acid (TFA) is added and the mixture is incubated at 37 °C for 24 hr. The cleavage is stopped by addition of 5 µl of 10% TFA. The solution is then transferred into a clean Eppendorf tube; the membrane pieces are washed with 100 µl of 10% TFA and the wash combined with the above solution.

Proteinase K cleavage. The procedure is the same as for trypsin except that buffers contain additionally 2M guanidinium hydrochloride and 50 ng of Proteinase K is used in place of trypsin. After 24 hr incubation an additional 50 ng of Proteinase K is added and the incubation continued for 3 hr.

The trypsin and Proteinase K cleavage products are separated on a reverse phase HPLC column (Aquapore RP-300, 1x100mm, C8, Brownlee/Applied Biosystems) according to standard procedures. The separated peptides are then analysed on an automated pulsed liquid microsequencing apparatus (Applied

Biosystems, Model 475A, Foster City, CA, USA) with automatic on-line amino acid analysis (Applied Biosystems, Amino Acid Analyser Model 120, Foster City, CA, USA). The following sequence information was obtained:

48kD Tocopherol Cyclase, Proteinase K cleavage

X-Leu-Ala-Pro-Val-Gln-(Ser)-Pro-
(Gly)-Leu-Asp-Leu-Ala-Pro-
X-Val-Gln-Leu-(Asp)-(Ser)-Asp-Gly-Glu-(Thr)-Val-
X-Leu-(Pro)-Val-

where X is an unknown amino acid and () indicates uncertainty.

50 kD tocopheron cyclase, direct N-terminal sequencing

H_2N-(Pro)-Leu-Tyr-Ile-Pro-X-Val-(Glu)-(Pro)-(Met)-(Tyr)-(Asp)-(Asp)-(Ala)-(Ala)-(Phe)-(Thr)

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: F. Hoffmann-La Roche AG
CH-4002 Basle / Suisse

(ii) TITLE OF INVENTION: Novel enzyme
(a Tocopherol Cyclase)

(iii) NUMBER OF SEQUENCES: 9

(vii) APPLICATION DATA:
(A) APPLICATION NUMBER: EP 92110874.2
(B) FILING DATE: 26-JUN-1992

(viii) REFERENCE: RAN 4213/114-001

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Chlorella protothecoides

(ix) FEATURE:
(A) NAME/KEY: Region (B) LOCATION: 9..16.. 17
(D) OTHER INFORMATION: residue numbers 9, 16 and17 below are uncertain, but are believed to be threonine, arginine and threonine, respectively.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ser Leu Tyr Asp Pro His Val Pro Thr Met Tyr Asp Pro Ala Phe Arg
1 5 10 15

Thr
17

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Chlorella protothecoides

(ix) FEATURE:
(A) NAME/KEY: Region (B) LOCATION: 3.. 10.. 14
(D) OTHER INFORMATION: residues 3, 10 and 14 are uncertain, but are believed to be alanine, arginine and lysine, respectively; Xaa being an amino acid not determinable

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Xaa Xaa Ala Val Tyr Val Ala Gln Leu Arg Gly Ile Gly Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Chlorella protothecoides

(ix) FEATURE:
(A) NAME/KEY: Region
(B) LOCATION: 1.. 3
(D) OTHER INFORMATION: residues 1 and 3 are uncertain, but are believed to be glycine, glycine and arginine, respectively;

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gly Ala Gly Leu Ala Arg Phe Glu
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Chlorella protothecoides

(ix) FEATURE:
(A) NAME/KEY: Region
(B) LOCATION: 13..15
(D) OTHER INFORMATION: residue numbers 13 and 15 in the sequence listing are uncertain, but each are believed to be glycine;
Xaa being an amino acid not determinable

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Xaa Asn Ala Leu Tyr Leu Ile Asp Leu Gln Tyr Thr Gly Gly Gly Xaa
1 5 10 15

Val Lys

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Chlorella protothecoides

(ix) FEATURE:
(A) NAME/KEY: Region

(B) LOCATION: 4..5
(D) OTHER INFORMATION: the amino acid at residue 4 in the below sequence is uncertain, however, it is believed to be arginine.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Gln Val Pro Arg Glu Ala Asn Asn
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Chorella protothecoides

(ix) FEATURE:
(A) NAME/KEY: Region
(B) LOCATION: 7..8
(D) OTHER INFORMATION: the amino acid at residue 7 in the below sequence is uncertain, however, it is believed to be serine;
Xaa being an amino acid not determinable

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Xaa Leu Ala Pro Val Gln Ser Pro
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Chlorella protothecoides

(ix) FEATURE:
(A) NAME/KEY: Region
(B) LOCATION: 1..2
(D) OTHER INFORMATION: the first amino acid residue in the below sequence is uncertain, however, it is believed to be glycine.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Gly Leu Asp Leu Ala Pro
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Chlorella protothecoides

(ix) FEATURE:
(A) NAME/KEY: Region
(B) LOCATION: 5.. 6.. 10
(D) OTHER INFORMATION: amino acids at residues 5, 6 and 10 below are uncertain, but are asparagine, serine and threonine, respectively; Xaa being an amino acid not deferminable

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Xaa Val Gln Leu Asp Ser Asp Gly Glu Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Chlorella protothecoides

(ix) FEATURE:
(A) NAME/KEY: Region
(B) LOCATION: 3..4
(D) OTHER INFORMATION: the amino acid at residue number 3 in the below sequence is uncertain, however, it is believed to be proline; Xaa being an amino acid not deferminable

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Xaa Leu Pro Val
1

Claims

1. Tocopherol cyclase in homogeneous form, which acts on phytyl benzoquinol derivatives to produce R',R',R'-tocopherols enantioselectively.

2. Tocopherol cyclase according to Claim 1, being of eukaryotic, preferably of algal or plant origin, e.g. from wheat leaves.

3. Tocopherol cyclase according to claim 1 or 2, and stemming from green algae, preferably Dunaliella salina or Chorella protothecoides, and being in the soluble fraction of the cells.

4. Tocopherol cyclase according to claim 1, 2 or 3, having the following properties:

molecular weight: 48 kD - 50 kD
and containing at least one of the following amino acid sequences:

H_2N-(Pro)-Leu-Tyr-Ile-Pro-X-Val-(Glu)-(Pro)-(Met)-(Tyr)-(Asp)-(Asp)-(Ala)-(Ala)-(Phe)-(Thr);
X-Leu-Ala-Pro-Val-Gln-(Ser)-Pro;
(Gly)-Leu-Asp-Leu-Ala-Pro;
X-Val-Gln-Leu-(Asp)-(Ser)-Asp-Gly-Glu-(Thr)-Val;
X-Leu-(Pro)-Val;
X being an amino acid residue not determinable and
( ) indicating uncertainty.

5. Tocopherol cyclase according to any one of Claims 1 to 4, having the following properties:

optimum pH range:	6,5-7
optimum temperature range:	25-35 ° C
stabilisation through:	glycerol, preferably ca. 8-12%

6. Tocopherol cyclase according to any one of Claims 1 to 5, producing R',R',R'-γ-tocopherol.

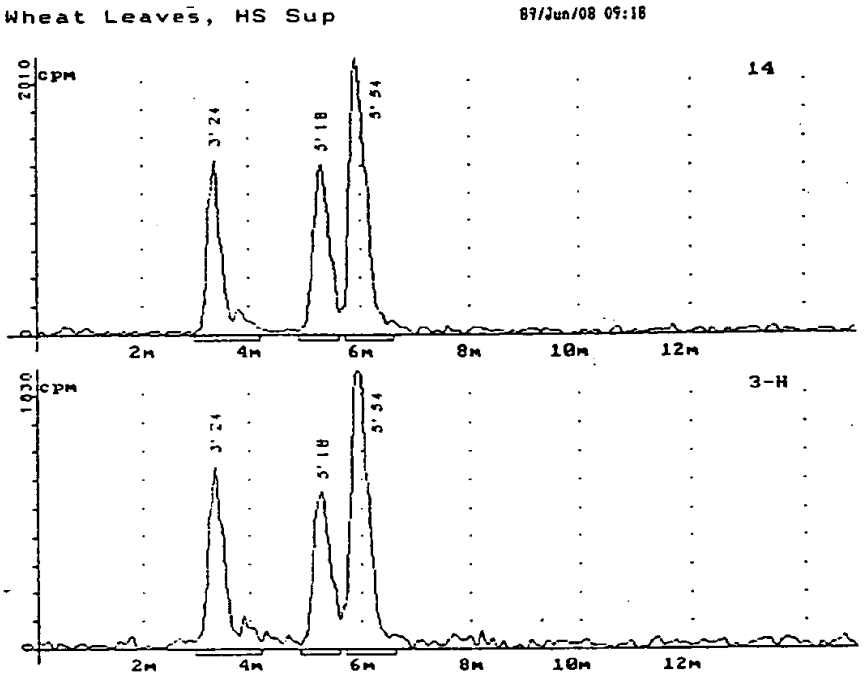
7. A process for producing the enzyme of any one of Claims 1 to 6, which comprises cultivating cells of an eukaryotic organism, preferably of green algae, said cells being capable of producing the novel tocopherol cyclase, disrupting the cells, isolating and purifying it from the soluble fraction of the cells.

8. A process according to Claim 7, wherein said algae represents the species Dunaliella salina or Chlorella protothecoides.

9. A process according to Claim 6, 7 or 8, involving essentially the following sequential purification steps:

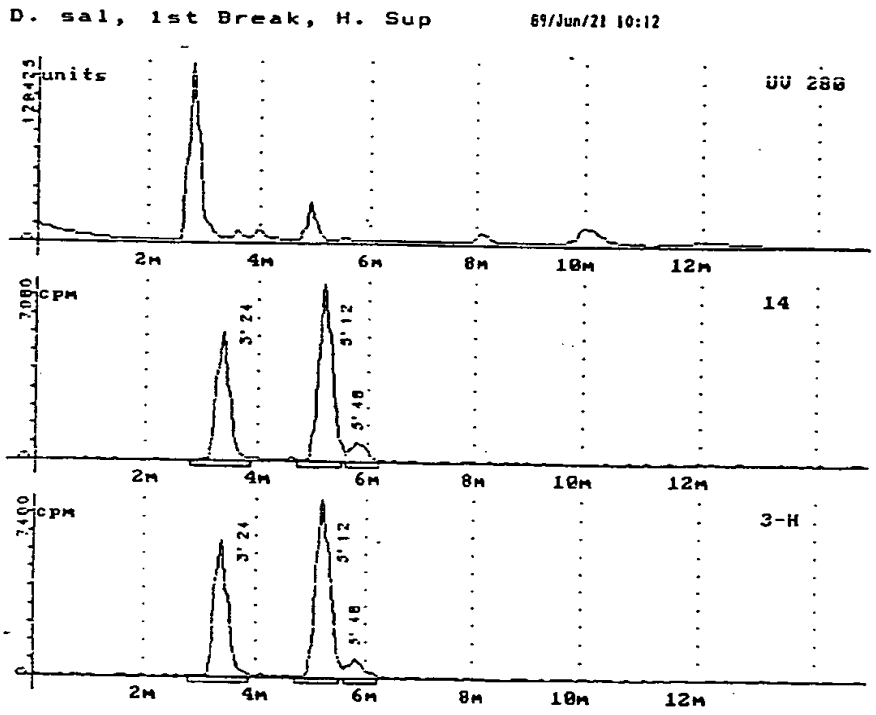
centrifugation
gel filtration
hydrophobic interaction chromatography
ion exchange chromatography
reverse phase chromatography.

10. Tocopherol cyclase in homogeneous form, obtainable by the process of Claim 7, 8 or 9.



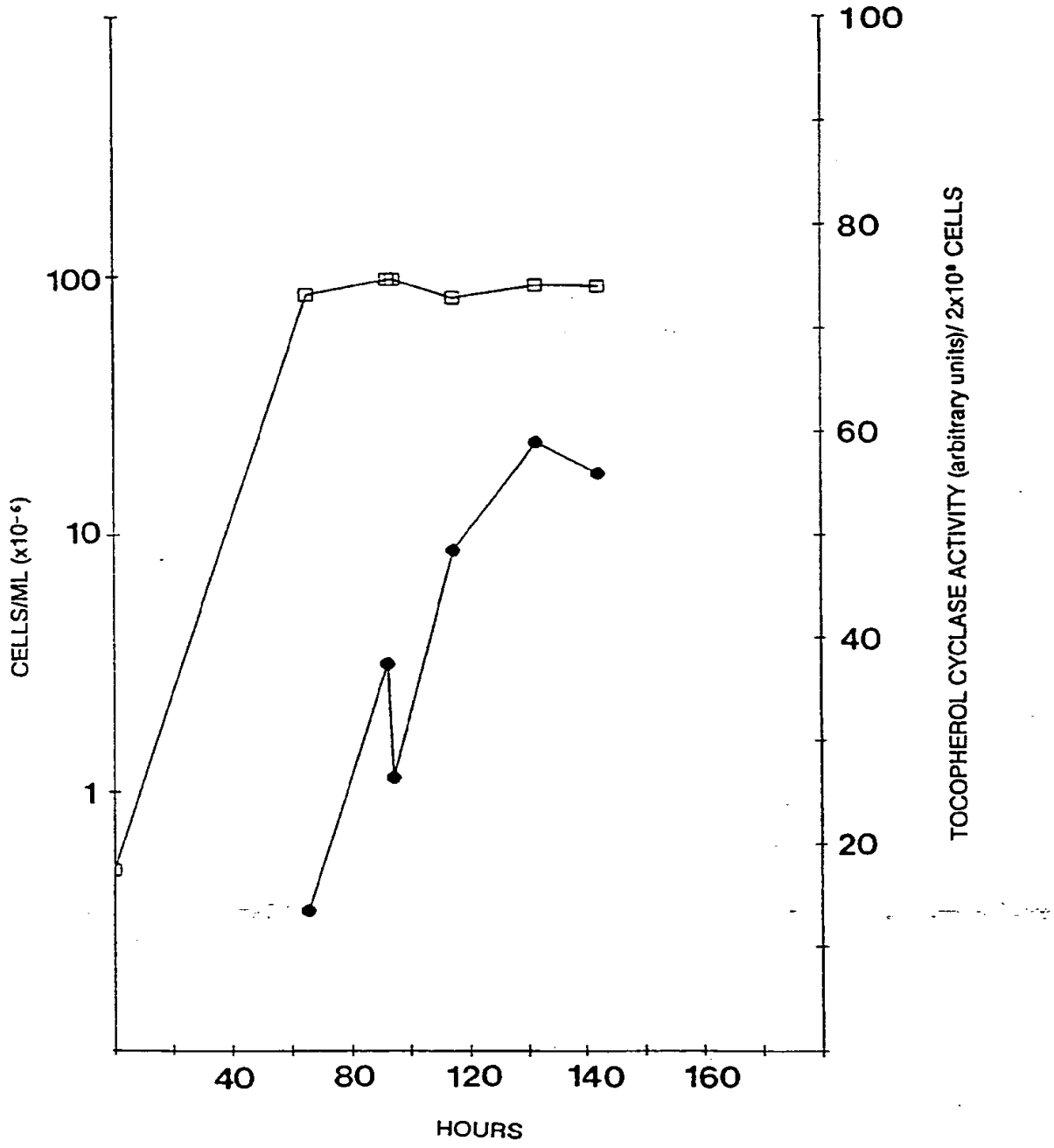
HPLC CHROMATOGRAM SHOWING THE SYNTHESIS OF (^{14}C,^{3}H)-GAMMA-TOCOPHEROL BY AN EXTRACT OF WHEAT LEAVES. 1ml of wheat leaf extract as prepared in Example 1 is incubated with the substrate (^{14}C, ^{3}H)-2,3-dimethyl-6-phytylhydroquinone. The existence of tocopherol cyclase activity is indicated by presence of (^{14}C,^{3}H)-gamma-tocopherol in the reaction mix. The HPLC chromatogram (see Example 1 for running conditions) shows two substrate peaks (3.2 and 5.5 min) and a gamma-tocopherol peak (5.2 min).

Figure 1



HPLC CHROMATOGRAM SHOWING THE SYNTHESIS OF (^{14}C,^{3}H)-GAMMA-TOCOPHEROL BY A CELL EXTRACT FROM DUNALIELLA SALINA. 1ml of cell extract as prepared in Example 2 is incubated with substrate (^{14}C,^{3}H)-2,3-dimethyl-6-phytylhydroquinone. The existence of tocopherol cyclase activity is indicated by the presence of (^{14}C,^{3}H)-gamma-tocopherol in the reaction mix. The HPLC chromatogram (see Example 2 for running conditions) shows two substrate peaks (3.2 and 5.5 min) and a gamma-tocopherol peak (5.1 min).

Figure 2



GROWTH VERSUS TOCOPHEROL CYCLASE ACTIVITY
(□—□ , cell count; ●—● tocopherol cyclase activity)

Figure 3

SDS-PAGE ANALYSIS OF ELUTION FRACTIONS FROM A PRO RPC COLUMN. Fractions eluted from a ProRPC column with a gradient of isopropanol (- - - - -) are assayed for tocoph rol cyclas activity (o——o)(abov) and analysed by SDS-PAGE (below). Activity correlates with two proteins, with molecular weights 48 and 50kD.

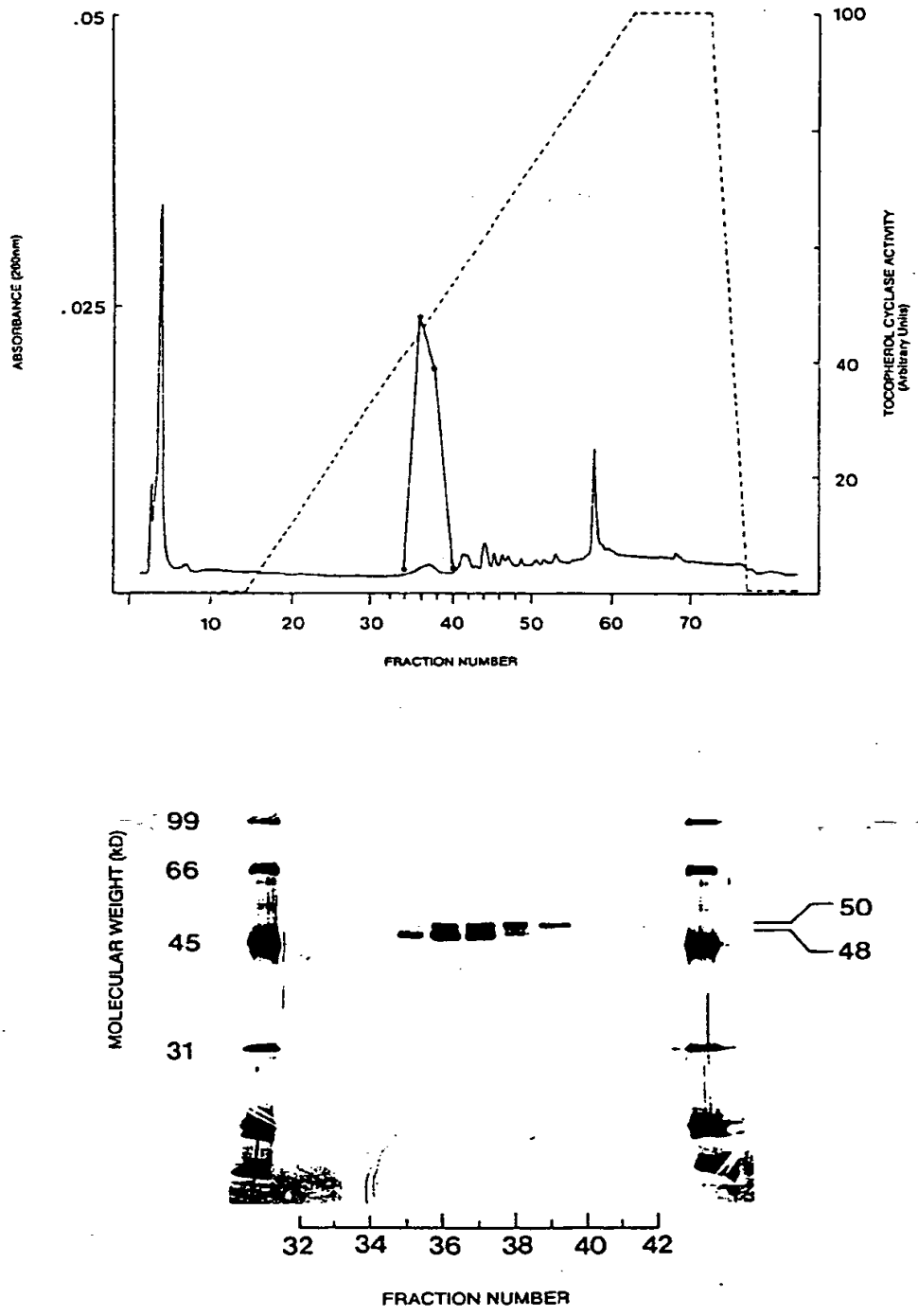


Figure 4

Amino acid	48	48 + 50
	residues/ mole	residues/ mole
Asx	42,1	39,4
Glx	46,3	45,9
Ser	20,0	23,3
His	6,9	8,2
Gly	81,2	93,0
Thr	29,4	31,4
Ala	40,7	43,5
Arg	22,6	23,8
Tyr	7,4	7,5
Cys-Cys	16,2	15,5
Val	29,0	29,1
Met	1,7	2,8
Ile	9,6	9,3
Phe	13,9	14,5
Leu	42,7	43,4
Lys	10,0	16,6
Pro	37,2	33,3

Amino Acid Analyses of 48 kD Tocopherol Cyclase and a Mixture of the 48 kD and 50 kD Tocopherol Cyclases.

Figure 5

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(54) Tocopherol cyclase.

(57) The invention concerns a novel enzyme, namely a cyclase, and more specifically, a tocopherol cyclase in homogeneous, pure form which acts on phytyl benzoquinol derivatives to produce R',R',R'-tocopherols enantioselectively.

EP 0 531 639 A3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 92 11 0874

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
A	CHEMICAL ABSTRACTS, vol. 110, no. 9, 27 February 1989, Columbus, Ohio, US; abstract no. 73888v, YAMAMOTO,YOSHIKAZU ET AL. 'Production of carbon-13 containing biomolecules by plant tissue culture.' page 507 ; * abstract * & JP-A-62 186 789 (NIPPON PAINT) 15 August 1987 ---	1	C12N9/88 //C12P17/06
A	CHEMICAL ABSTRACTS, vol. 99, no. 21, 21 November 1983, Columbus, Ohio, US; abstract no. 174542, AGENCY OF INDUSTRIAL SCIENCES AND TECHNOLOGY 'Tocopherols and tocotrienols from cereal plant wastes.' page 516 ; * abstract * & JP-A-5 899 475 (...) 13 June 1983 ---	1	
A	EP-A-0 058 945 (EISAI) 1 September 1982 * claims * ---	1	TECHNICAL FIELDS SEARCHED (Int.Cl.5) C12N C12P C07D
A	WO-A-90 01554 (HOFFMANN LA ROCHE) 22 February 1990 * claims * -----	1	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	24 February 1994	Delanghe, L

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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